Non-naturally Occurring Lipoprotein Particle

CROSS-REFERENCES TO RELATED APPLICATIONS

This application is a continuation-in-part of copending U.S. Patent Application No. 09/269,533, filed on June 1, 1999,
5   which is a national phase application of PCT/GB97/02610, filed on September 25, 1997, which claims priority from application G9620153.8, filed on September 27, 1996, which are hereby incorporated herein in their entirety by reference.

10   BACKGROUND

The present invention relates to lipoprotein particles, a process for preparing such particles and their use. In particular, the invention relates to non-naturally occurring low density lipoprotein particles, methods for their
15   manufacture and use thereof.

Low density lipoprotein (LDL) is a natural component of plasma which is involved in the transport of cholesterol in the form of cholesterol esters around the body. Naturally occurring LDL is known to occur as roughly spherical-shaped
20   particles (20-22 nm in diameter) which comprise an internal core of about 1500 cholesterol esters containing small amounts of triglyceride (TG). The internal core is typically surrounded by a solubilising monolayer of about 800 phospholipid molecules and small quantities of free cholesterol
25   (about 500 molecules). Located in the monolayer is a large receptor protein, Apo protein B, of approx 500,000 daltons, (Apo B) which accounts for about twenty percent of the weight of the LDL particle.

Naturally occurring LDL finds use in a number of areas,
30   for example, in studies on atherosclerosis and lipid metabolism. LDL also finds use as a drug-targeting vector in

cancer chemotherapy. Certain cancerous cells display high rates of receptor mediated LDL uptake relative to normal cells and as such, LDL has also found use as a targeting vector for anti-cancer drugs.

5   Currently, naturally occurring LDL needs to be isolated from fresh plasma samples. The isolation procedure is lengthy (e.g. up to 48 hours) and depending on the donor's plasma LDL levels the yield of LDL can be any amount up to about 100mg ApoB/100ml plasma in healthy individuals. Thus, yields are 10   generally low.

Isolated naturally occurring LDL is known to be unstable. Attempts have been made to produce LDL-like particles, generally in the form of microemulsions of similar size and lipid composition to naturally occurring LDL, however such 15   particles lack receptor competency. Apo B may be grafted onto such microemulsion particles, however, the grafting process still requires a source of the protein from fresh plasma.

Apo B is difficult to graft onto microemulsion particles partly because of its large size and a tendency for it to 20   aggregate due to its amphipathic character. As such, grafting of Apo B onto microemulsion particles is not satisfactory because of *inter alia* inherent problems associated with the grafting-on process, and instability of the Apo B component.

It has now been found that a non-naturally occurring LDL 25   can be produced which possesses LDL receptor competency, yet does not require the use of substantially whole Apo B or substantially whole analogues thereof. Furthermore, a process for the production of non-naturally occurring LDL has been developed which does not require the use of plasma derived LDL 30   and/or plasma derived Apo B.

SUMMARY OF INVENTION

An object of the present invention is to provide a non-naturally occurring LDL possessing Apo B receptor competence.

Another object of the present invention is to provide a

5   process for producing non-naturally occurring LDL particles.

These and other objects of the invention will become apparent from the following description and examples.

STATEMENT OF INVENTION

10   According to a first aspect of the present invention there is provided a non-naturally occurring, receptor competent LDL particle comprising at least one peptide component wherein the said peptide component comprises at least a binding site for an Apo B protein receptor and at least one lipophilic substituent.

15   A non-naturally occurring LDL particle is one which is not found occurring naturally *in vivo*. A non-naturally occurring LDL must be receptor competent i.e. capable of binding to Apo B receptors and/or capable of eliciting an Apo B protein-like physiological effect on and/or after binding. Thus, the non-

20   naturally occurring LDL particle comprises at least a sequence of amino acids such as a protein, polypeptide or peptide capable of binding to Apo B receptors, which polypeptide may or may not be identical in respect of its binding region with the amino acid sequence of an Apo-B binding site, for example, an

25   Apo B 100 binding site or physiologically functional peptide analogues thereof. Naturally, the skilled addressee will appreciate that the polypeptide capable of binding to Apo B receptors on target cells, such as cancer cells expressing Apo B receptors, is able to elicit an Apo B protein-like

30   physiological effect on and/or after binding i.e. to be receptor competent.

The LDL particle comprises at least two components, a lipid component (L-component) and a peptide component (P-

component). The L-component generally comprises a lipid emulsion comprising a core of lipophilic molecules such as cholesteryl esters, for example, cholesterol oleate, cholesterol linoleate, cholesterol stearate and the like.

5   Other suitable lipophilic core molecules can comprise triglycerides, for example, triolein, plant oils such as soya bean oil and even lipophilic drugs, for example, estramustine, prednimustine and lipophilic modifications of known drugs, such as anti-cancer drugs, for example, cholesteryl esters of

10   methotrexate and the like. The core of the L-component is typically solubilised by a lipid, such as an amphiphilic lipid comprising a charged or hydrophilic group. Such amphiphilic lipids include unesterified cholesterol and suitable non-ionic surfactants as well as phospholipids such as phosphatidyl

15   choline, sphingomyelin and phosphatidyl glycerol. Preferably, the cholesteryl esters are solubilised by a monolayer of phospholipid. The preparation of the L-component is known in the art and may be performed using a variety of methods as described in the art, e.g. Ginsburg, G.S. et al (1982) J. Biol.

20   Chem 257 (14) pp 8216-8227; Owens M.D. and Halbert G.W. (1993) J. Pharm. Pharmacol. 45 (Suppl.) p68P; Owens M.D. and Halbert G.W. (1995) Eur. J. Pharm. Biopharm 41 (2) pp 120-126, herein incorporated by reference.

     Preferably, the L-component is made up of at least two

25   biologically acceptable components. A first component can be a biologically acceptable saturated or unsaturated long chain charged polar component such as a phospholipid. Examples of suitable charged polar components include phosphatidyl choline (PC), phosphatidyl serine (PS), phosphatidyl glycerol (PG),

30   sphingomyelin, and unesterified cholesterol and the like. The second component can be a biologically acceptable lipophilic component such as a cholesteryl ester, for example cholesteryl oleate. Biologically acceptable components are ones which may

be administered to cells *in vitro* or *in vivo* and which have substantially no deleterious effect on cell viability. In a preferment the L-component can comprise three or more components in a defined ratio, such as a molar ratio, for example, phospholipid; triolein; cholesteryl ester (P:T:C). The molar ratio may be in any molar ratio as long as the components are capable of forming an L-component suitable for use in the preparation of non-naturally occurring LDL particles of the present invention. The molar ratio of outer core solubilising lipid such as phospholipid (PL), sphingomyelin (SM), phosphatidyl choline (PC) and unesterified cholesterol (UC) to core lipid such as cholesteryl ester (CE), triolein (TO) cholesteryl oleate (CO) or lipophilic drug can be in the range of from about 0.7:1 up to 5:1, preferably 1:1 to 3:1 depending on design. A preferred ratio of PL:CE is about 2:1. Where a third L-component is not employed the ratio of PL:CE can be in the range of from about 1:1 to about 2:1. A suitable molar ratio for a three component system such as a phosphatidylcholine: triolein: cholesteryl oleate is 3:2:1 respectively.

A suitable molar ratio for a five component system comprising three outer core lipids and two core lipids may lie in the range of from 0.7-6.5: 0-2:0-1 (outer core lipid): 0-5: 0-2.5 (core lipid). Preferably, the molar ratio lies in the range of from 2.5-4.5: 1-2: 0.5-1 (outer core lipid): 2-4.5: 1-2.5 (core lipid). More preferably the molar ratio lies in the range of from 4-4.5: 1.5-2: 0.7-0.9 (outer core lipid): 4-4.5: 1.8-2.2 (core lipid). Suitable outer core lipids may be selected from PC, SM, UC and PL. Suitable core lipids may be selected from TO, CE and CO. The man skilled in the art will appreciate that other suitable outer core lipids and core lipids may be used in the present invention. An example of a five component system is PC: SM: UC (outer core lipid): TO: CO

(core lipid). The components of such a five component system may be present in molar ratios as indicated above.

Generally, the droplet diameter of lipid microemulsions employed in the non-naturally occurring lipoprotein particles
5   of the invention should be capable of functioning as lipoprotein particles *in vivo*, *ex vivo* or *in vitro*. The diameter of the non-naturally occurring LDL particles can be up to about 50 nm, preferably from about 10 nm up to about 35 nm depending on the method of preparation and/or molar ratio such
10   as a PL:CE molar ratio, employed.

Peptide components for use in forming LDL particles of the invention contain at least one lipophilic substituent or moiety capable of acting as an "anchor" for anchoring the peptides to the L-component. Lipophilic moieties or substituents may be
15   derived from biologically compatible lipophilic compounds such as cholesterol, retinoic acid, C_{10}-C_{22} fatty acids such as stearic acid (C_{18}) and the like including the components already described which make up the L-component or part thereof of the LDL particle such as a biologically acceptable lipophilic
20   component e.g. cholesteryl esters, for example cholesteryl oleate, triolein or a lipophilic drug. As described herein, biologically acceptable components are ones which may be administered to cells *in vitro* or *in vivo* and which have substantially no deleterious effect on cell viability.

25   Other lipophilic components which may be used as a lipophilic substituent or moiety for anchoring the peptides to the L-component may be biologically acceptable or may be cytotoxic. Further examples of lipophilic substituents include the following compounds or derivatives thereof which may be
30   attached to the N- and/or C-terminus of the peptide component: Lipid soluble cytotoxic drugs, e.g. etoposide and methotrexate diester; pyrenes or compounds derived therefrom e.g. pyrene butyric acid, benzo(a)pyrene, 3-hydroxybenzo(a)pyrene and

benzo(*a*)pyrene-7,8-dihydrodiol; retinyl derived compounds e.g. *N*-retinoyl-L-leucyl DOX-14-linoleate; polyunsaturated compounds, e.g. β-carotene; hormones e.g. estradiol, testosterone and aldosterone and the like; diphenylhydantoin;

5   bishydroxycoumarin; pentobarbital; perfluorinated cholesteryl oleate; anthracycline AD-32; PCMA cholesteryl oleate.

These and other suitable lipophilic compounds are described in Chapter 4 Lipoproteins and Microemulsions as Carriers of Therapeutic and Chemical Agents by Florence &

10   Halbert in the book Lipoproteins as Carriers of Pharmacological Agents Ed. J. Michael Shaw, Publisher Marcel Dekker, Inc., which is incorporated herein by reference in its entirety.

The lipophilic moiety/substituent can be placed in contact with the amino and/or carboxy terminus of the peptide via

15   chemical means such as covalent bonding or ionic bonding known in the art. The man skilled in the art will appreciate that peptides of the invention can be assembled using standard Fmoc protocols of the Merrifield solid phase synthesis method. The lipophilic substituent, such as retinoic acid can be activated

20   and attached to the peptide N-terminus using a standard peptide coupling cycle. Initially an acid labile linker such as 3-methoxy-4-hydroxymethylphenoxyacetic acid is attached to the resin support and esterified with the first amino acid (C-terminus) of the target peptide. When peptide assembly is

25   complete the ester to the linker can be hydrolysed, allowing removal of the fully protected peptide, for example with trifluoroacetic acid (TFA) eg. 1% TFA, in dichloromethane which can subsequently be evaporated off. At such a stage, the available functional group is the peptide carboxyl, which can

30   be activated with for example one equivalent of dicyclohexylcarbodiimide (DCC) in dimethylformamide (DMF) and coupled to a lipophilic molecule, such as cholesterol (10 equiv), to yield ester. Evaporation of the solvent and

treatment with TFA, e.g. 95% TFA, deprotects the amino acid side chains, completing the synthesis. The complete peptide can then be concentrated and precipitated with, for example, diethyl ether to give a solid which can then be washed as necessary to remove any remaining protecting group fragments and excess cholesterol.

N-terminal modifications, such as retinoic acid and stearate addition, targeted at primary amines can be used in the synthesis of modified peptides of the invention using techniques known in the art. Preferably, peptides capable of being utilised in the invention are amphipathic in nature, i.e. possess lipophilic and hydrophilic groups. Suitable hydrophilic groups include hydroxyl, carboxylic and amino groups. Where the peptides are amphipathic in character, the hydrophobic group and hydrophilic groups may be located at any suitable point thereon via appropriate side chains. Preferably the hydrophobic groups and hydrophilic groups are located either at the amino terminus and carboxy terminus of the peptide respectively or vice versa.

In an aspect of the invention there is provided non-naturally occurring LDL particles comprising peptides wherein the amino acid sequence of the binding site of said peptides are selected from the group: amino acids having basic side chains, amino acids having aliphatic side chains, and amino acids having aliphatic hydroxyl side chains.

In this aspect of the invention, the peptides of the non-naturally occurring LDL of the invention may display substantial, little or no similarity and/or identity with the amino acid sequence of the Apo B binding region. Preferably, the peptides display substantial similarity and/or identity with the amino acid sequence of the Apo B binding region.

The amino acid sequence which makes up the peptide capable of being grafted onto the lipid component of the LDL of the

present invention can be selected from the group of amino acids having basic side chains e.g. lysine, arginine and histidine; amino acids having aliphatic side chains e.g. glycine, alanine, valine, leucine and isoleucine; amino acids having aliphatic

5   hydroxyl side chains e.g. serine and threonine, and derivatives thereof.

Where the binding region amino acid sequence is substantially dissimilar to the binding region sequence of Apo B with respect to the order of amino acids incorporated

10   thereinto, the amino acids selected for inclusion into the binding region of the amino acid sequence can be selected from substantially the same amino acids as those making up the Apo B binding region sequence. Naturally, the skilled addressee will understand that conservative replacement and/or

15   substitutions as herein described may also be made to such binding regions.

In a preferment there is provided a non-naturally occurring LDL particle comprising at least one amino acid sequence including an Apo B binding region capable of

20   interacting with an Apo B receptor.

Naturally, the skilled addressee will appreciate that such amino acid sequences making up functional peptides or polypeptides suitable for use in the present invention must be receptor competent as defined herein. Thus, synthetic or semi-

25   synthetic peptides and/or polypeptides and analogues thereof capable of binding to Apo B receptors are encompassed by the present invention.

In a preferment, the amino acid sequence can comprise any one, two or all of the Apo B binding site sequence(s) depicted

30   below in the same peptide or in the form of dimers or in different peptides:

(1) Lys Ala Glu Tyr Lys Lys Asn Lys His Arg His (SEQ ID NO: 1);

or

(2) Thr Thr Arg Leu Thr Arg Lys Arg Gly Leu Lys (SEQ ID NO: 2);

or

(3) Arg Leu Thr Arg Lys Arg Gly Leu Lys (SEQ ID NO: 8),

and analogues thereof which are capable of binding to the Apo B100 receptor site.

The amino acid sequence can be of any length provided that it is capable of being grafted onto the lipid component under grafting conditions as described herein.

The amino acid sequence may include sequences of up to about 500 amino acid residues long comprising sequences (1) and/or (2) and/or (3) above. Sequences (1), (2) and (3) are known Apo B binding site sequences identified from the human Apo-100 protein as described by Knott T.J. et al Nature Vol. 323 October 1986 p 735. For example, an amino acid sequence could comprise the sequence from amino acid 3079 to about position 3380 of Fig 1, p 735 (Knott et al supra). The amino acid sequence can comprise at least a single Apo B binding site sequence and can be from about 8-200 amino acid residues in length, or a shorter sequence of from about 8-50 amino acid residues in length, preferably from about 9 to 30 amino acid residues in length. Examples of suitable peptide sequences include those as depicted in Figure 7 herein. Naturally, the skilled addressee will appreciate that practical considerations such as the ability of the amino acid sequence to bind to receptor and ability to synthesise the peptide sequence generally means that the shorter amino acid sequences are preferred. The skilled addressee will appreciate that natural variations in the amino acid sequences comprising amino acid

substitutions, deletions and/or replacements are encompassed by the present invention. Furthermore, the skilled addressee will also appreciate that amino acid substitutions, deletions and/or replacements can be made to the amino acid sequence so long as

5   such modifications do not substantially interfere with the ability of the amino acid sequence to bind to a binding site and thereby elicit a physiological response. For example, conservative replacements may be made between amino acids within the following groups:

10   (i)   Lysine and arginine;

(ii)   Alanine, serine and threonine;

(iii)   Glutamine and asparagine;

15

(iv)   Tyrosine, phenylalanine and tryptophan; and

(v)   Leucine, isoleucine, valine and methionine.

20   so long as the physiological function of the peptide is not substantially impaired.

The invention further includes substantially isolated proteins having a substantially similar activity/function to the peptides of the instant invention and which have an amino

25   acid sequence which comprises at least 70% similarity, that is, identity, to the sequence of peptide (1) and/or peptide (2) and/or peptide (3) above when aligned optimally therewith. It is preferred that such a degree of similarity, that is identity, is at least 80%, more preferred that such a degree of

30   similarity is 90% or higher. In the context of the present invention two amino acid sequences with at least 70% similarity to each other have at least 70% identical or conservatively replaced amino acid residues in a like position when aligned

optimally using computer programs known in the art, such as
BLAST and FASTA.

In a further aspect of the invention there is provided use
of non-naturally occurring LDL particles as drug-targeting
5   vectors.   Such drug targeting vectors can be used in the
treatment of cancerous cells and the like.

In another aspect of the invention there is provided a
process of producing a non-naturally occurring, receptor
competent LDL particle possessing Apo B receptor competency
10   which comprises:

i)   forming a lipid component from lipophilic molecules and
        amphiphilic lipid molecules; and

ii)   contacting at least one peptide with a lipid component
15   formed in i) wherein the at least one peptide comprises
        at least a lipophilic substituent and at least a binding
        region capable of interaction with an apoprotein B
        receptor.

Preferably the process of producing a non-naturally
20   occurring receptor competent LDL particle possessing ApoB
receptor competency comprises:

i)   forming a lipid and peptide mixture by dissolving
        lipophilic molecules, amphiphilic lipid molecules
25   and a peptide in a lipophilic dissolving solvent;
ii)   contacting and mixing the mixture formed in i) with
        an aqueous phase containing an emulsifier.

The peptide is as defined herein above.
30   The solvent in step i) may be any solvent capable of
dissolving lipophilic components for example dichloromethane.

The emulsifier may be any emulsifier known in the art, for
example, sodium oleate.

The mixing step may be performed at a high shear to obtain good mixing of the two phases, for example a sonication technique and/or a microfluidisation technique.

Following mixing, the solvent(s) may be removed, e.g. by evaporation such as under reduced atmospheric pressure.

Additionally, a fluorescent probe may be incorporated into the lipophilic particles by including the probe in the lipophilic dissolving solvent.

A suitable fluorescent probe is e.g. 3,3'-dioctadecyloxacarbonocyanine perchlorate (DiO) which may be used at a concentration of about 0.08 mg/ml in the said solvent mixture, e.g. at a concentration of 0.079 mg/ml.

In a further aspect of the invention there is provided use of an nLDL particle as a drug targeting vector, in particular in the treatment of cancerous cells comprising Apo B protein receptors in a patient.

In a still further aspect of the invention there is provided use of an nLDL particle in the manufacture of a medicament for the treatment of disease, in particular cancer. Thus, there is provided use of an nLDL particle in the manufacture of a drug targeting vector comprising a medicament therein for the treatment of cancerous cells comprising Apo B protein receptors.

The skilled addressee will appreciate that non-naturally occurring, receptor competent LDL particles of the invention may be administered to a patient along with conventional vehicles or carriers typically used in drug and vesicle presentation such as water, physiologically acceptable saline solutions, buffers and the like.

As a further aspect of the invention there is provided a pharmaceutical formulation comprising a non-naturally occurring receptor competent LDL together with a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers may

be selected from conventional carriers commonly employed in the art.

Additionally, there is provided a method for the treatment of cancer in a patient which comprises administering to the patient a clinically useful amount of a non-naturally occurring LDL particle comprising an anti-cancer drug.

The lipid component is as hereinbefore described as are the types of peptides which may be employed.

A further aspect of the present invention provides use of a non-naturally occurring low density lipoprotein particle as hereinbefore described in a cell culture medium, such as for example as a supplement for cell growth.

BRIEF DESCRIPTION OF THE DRAWINGS

The following Figures are provided to illustrate the present invention, and should not be construed as limiting thereof.

Figure 1: Proliferation of U937 Cells in RPMI supplemented with Different Lipid Sources.

Legend: Mean $\pm$ standard deviation (n=3). Cells were seeded at 1 x 10^5 in 75 cm^2 flask containing 25ml of RPMI supplemented with: $\square$ Serum 10% v/v, $\blacksquare$ Delipidated Foetal Calf Serum 5% v/v, $\blacktriangle$ No additions, $\blacklozenge$ LDL, $\bullet$ Control microemulsion. LDL and control microemulsion were added at an equivalent cholesterol concentration (0.08mmol/l) to Foetal Calf Serum. At 24 hour intervals, 0.5 ml of cell suspension was removed and counted.

Figure 2: Proliferation of U937 induced by Different Lipid Sources.

Legend: Mean $\pm$ standard deviation (n=3). Result expressed as increase in cell number after 72 hours relative to

mean result of control media with no additions. Microemulsion (ME) without peptide. Cholesterol concentration 0.08mmol/l, Peptide concentration 0.03mol/mol cholesterol oleate. No DFCS was used in any of the experiments. + No significant
5   difference p>0.05. # Significant difference p<0.05

Figure 3: Effect of Cholesterol Concentration and Delipidated Serum on the Growth of U937 Cells Supplemented with nLDL.

      Legend: Mean (n=2). Result expressed as increase in cell
10   number after 72 hours relative to mean result of control media with no additions. Open symbols {Peptide D} nLDL without DFCS, filled symbols {Peptide D} nLDL supplemented with DFCS 5% v/v. Peptide concentration 0.03mol/mol cholesterol oleate.

15   Figure 4a: Growth of U937 Cells Induced by Different nLDL Preparations.

      Legend: Figure 4a. Mean ± standard deviation (n=8). Result expressed as increase in cell number after 72 hours relative to mean result of control media with no additions.
20   Peptide concentration 0.03mol/mol cholesterol oleate. □ Microemulsion control; ◊ [Peptide A] nLDL; O [Peptide B] nLDL; ▲ [Peptide C] nLDL; ⊞ [Peptide D] nLDL; * [Peptide E] nLDL; ● LDL. All supplemented with DFCS 5% v/v.

25   Figure 4b: As for Figure 4a, no standard deviations plotted.

Figure 5: Effect of Anti-LDL Receptor Antibody on the Growth of U937 Cells Induced by different nLDL preparations.

      Legend: Mean ± standard deviation (n=4). Result
30   expressed as increase in cell number after 72 hours relative to mean result of control media with no additions. All supplemented with DFCS 5% v/v. Cholesterol concentration 0.026 mmol/l, peptide concentration 0.03mol/mol cholesterol oleate.

Significantly less than antibody control (p<0.05).

<u>Figure 6:</u>   Effect of increasing concentrations of Anti-LDLReceptor Antibody on the Growth of U937 Cells induced by {Peptide B} nLDL.

Legend: Mean ± standard deviation (n=4).   Result expressed as increase in cell number after 72 hours relative to mean result of control media with no additions.   All supplemented with DFCS 5% v/v.   Cholesterol concentration 0.033mmol/l, peptide concentration 0.03mol/mol cholesterol oleate.   # Significant difference (p<0.05).

<u>Figure 7:</u>   Synthesised peptide analogues of the Apo-B 100 binding site (Peptides A-E inclusive).

<u>Figure 8(a) and 8(b):</u>   The effect of increasing peptide concentrations on the Number Mean Diameter and Zeta Potential of various nLDL preparations.

Legend: Figure 8(a) and 8(b). Open symbols Mean Number Diameter, closed symbols Zeta Potential. ☐ [Peptide A]-nLDL (PC:TO:CO, 3:2:1); o [Peptide B]-nLDL (PC:UC:TO:CO, 6.2:1:4.2:2.1);   △   [Peptide   C]-nLDL   (PC:UC:TO:CO, 6.2:1:4.2:2.1). Mean ± standard deviation, n=10.

<u>Figure 9(a):</u> The effect of pH on the measured Zeta Potential of various [Peptide A]-nLDL preparations.

Legend: Figure 9(a). All preparations PC:TO:CO, 3:2:1. ■ DMSO treated control; ☐ 0.02 moles Peptide A/mole CO; ◊ 0.039 moles Peptide A/mole CO; o 0.106 moles Peptide/mole CO; △ 0.532 moles Peptide A/mole CO. Mean ± standard deviation, n=10.

Figure 9(b): The effect of pH on the measured Zeta Potential of various nLDL preparations.

Legend: Figure 9(b). All preparations PC:TO:CO, 3:2:1. ■ DMSO treated control; □ [Peptide A]-nLDL 0.119 moles/mole CO; ◊ [Peptide B]-nLDL 0.112 moles/mole CO; o [Peptide C]-nLDL 0.111 moles/mole CO. Mean ± standard deviation, n=10.

Figure 10(a): The effect of microemulsion formulation on the measured Mean Number Diameter and Zeta potential of [Peptide A]-nLDL preparations.

Legend: Figure 10(a). Open symbols Mean Number Diameter, closed symbols Zeta Potential. □ PC:TO:CO, 3:2:1, o PC:SM:TO:CO, 2.5:1:2.4:1.2; Δ PC:SM:UC:TO:CO, 4.4:1.8:1:4.2:2.1. Mean ± standard deviation, n=10.

Figure 10(b): The effect of microemulsion of formulation on the measured Mean Number Diameter and Zeta Potential of [Peptide B]-nLDL preparations.

Legend: Figure 10(b). Open symbols Mean Number Diameter, closed symbols Zeta Potential. □ PC:UC:TO:CO, 6.2:1:4.2:2.1; o PC:SM:UC:TO:CO, 4.4:1.8:1:4.2:2.1. Mean ± standard deviation, n=10.

Figure 10(c): The effect of microemulsion formulation on the measured Mean Number Diameter and Zeta Potential of [Peptide C]-nLDL preparations.

Legend: Figure 10(c). Open symbols Mean Number Diameter, closed symbols Zeta Potential. □ PC:UC:TO:CO, 6.2:1:4.2:2.1; o PC:SM:UC:TO:CO, 4.4:1.8:1:4.2:2.1. Mean ± standard deviation, n=10.

Figure 11: Total binding curves for various 14C-nLDL preparations to B16 cells *in vitro* at 4°C.

Legend: Figure 11. Cells were serum starved overnight and cooled to 4°C before the addition of media (also cooled to 4°C) supplemented with DFCS and containing nLDL preparations at a cholesterol concentration of 0.08mmol/l. All peptide concentrations were 0.03 mol/mol cholesterol. □ [NP]-nLDL; ◊ [Peptide A]-nLDL; o [Peptide B]-nLDL; △ [Peptide C]-nLDL; ⊞ [Peptide D]-nLDL; + [Peptide E]-nLDL.

Figure 12(a) and 12(b): Non-specific (Figure 12a) and specific (Figure 12b) binding curves for various 12C-nLDL preparations to B16 cells *in vitro* at 4°C.

Legend: Figure 12(a) and 12(b). Cells were serum starved overnight and cooled to 4°C before the addition of media (also cooled to 4°C) supplemented with DFCS and containing nLDL preparations at a cholesterol concentration of 0.08mmol/l. All peptide concentrations were 0.03 mol/mol cholesterol. Values for non-specific binding obtained were measured using an excess of unlabelled nLDL, specific binding values calculated by subtracting non-specific binding from total binding. □ [NP]-nLDL; ◊ [Peptide A]-nLDL; o [Peptide B]-nLDL; △ [Peptide C]-nLDL; ⊞ [Peptide D]-nLDL; + [Peptide E]-nLDL.

Figure 13: Double reciprocal plots of total binding data for various 14C-nLDL preparations to B16 cells *in vitro* at 4°C.

Legend: Figure 13. Experimental conditions as per Figure 12(a) and 12(b). Lines determined by linear regression, values calculated from data presented in Table 1. □ [NP]-nLDL; ◊ [Peptide A]-nLDL; o [Peptide B]-nLDL; △ [Peptide C]-nLDL; ⊞ [Peptide D]-nLDL; + [Peptide E]-nLDL.

Figure 14: Chromatography of DiO labelled nLDL after incubation with Bovine Serum Albumin.

Legend: Figure 14. ■ DiO; o Bovine Serum Albumin. Sephacryl S-300 column (bed volume 9.1 ml). DiO labelled nLDL was mixed with PBS/BSA (2 mg/ml) and incubated at 37°C before being added to column pre-equilibrated with PBS. Eluted fractions were analysed for DiO and protein content as described in methods. Values are single measurement of each fraction.

Figure 15: Dose response of DiO labelled nLDL binding to B16 cells.

Legend: Figure 15. Cells incubated at 4°C for three hours, mean (n = 5, observations varied by less than 16%), non-specific binding was not accounted for. ■ Microemulsion control; ● [Peptide A]-nLDL; ▲ [Peptide C]-nLDL.

Figure 16: Binding Characteristics of DiO labelled microemulsion and nLDL to B16 cells at 4°C.

Legend: Figure 16. Cells incubated at 4°C for three hours, mean (n = 5, observations varied by less than 16%). Specific High Affinity Binding was determined by subtracting non-specific binding (determined using a 100 fold excess of unlabelled ligand) from total binding. ■ Microemulsion total binding; □ microemulsion non-specific binding; ⊞ microemulsion specific binding; ● [Peptide A]nLDL total binding; O [Peptide A]nLDL non-specific binding; ⊕ [Peptide A]nLDL specific binding.

Figure 17: Binding and Internalisation of DiO labelled microemulsion and nLDL to B16 cells at 37°C.

Legend: Figure 17. Cells incubated at 37°C for three hours, mean (n = 5, observations varied by less than 16%). Specific High Affinity Binding was determined by subtracting

non-specific binding (determined using a 100 fold excess of unlabelled ligand) from total binding. ■ Microemulsion total binding; Δ microemulsion internalisation; ● [Peptide A]nLDL total binding; ▲ [Peptide A]nLDL internalisation.

Figure 18(a) and 18(b): Scatchard and Double Reciprocal Plots of DiO labelled microemulsion and nLDL binding to B16 cells at 4°C.

Legend: Figure 18(a). Scatchard Analysis of total binding at 4°C after three hours, mean (n = 5). ■ Microemulsion; ● [Peptide A]nLDL; ▲ [Peptide C]nLDL.

Legend: Figure 18(b). Double Reciprocal Analysis of total binding at 4°C after three hours, mean (n = 5). Symbols as Figure 18(a).

Figure 19: The effect of ethinyl estradiol on [Peptide]nLDL U937 cell proliferation.

Legend: Figure 19. Mean ± standard deviation n = 4. Results expressed as increase in cell number after 72 hours relative to mean result of control media with no additions. Open symbols no ethinyl estradiol, closed symbols ethinyl estradiol (2μg/ml). □ [Peptide A]nLDL; o [Peptide B]nLDL. Cholesterol concentration 0.08 mmole/l.

Figure 20: The effect of the pyrene butyric acid peptide on NSO cell proliferation.

Legend: Figure 20. Proliferation of NSO cells induced by sLDL containing peptide F (0.03) after 72 hours incubation. Level of peptide present in sLDL varies from 0.03 moles/mole of cholesterol ester. sLDL lipid constituents: cholesterol, cholesterol oleate, triolein, phospholipid and oleic acid.

Figure 21: The effect of the pyrene butyric acid peptide on HFFF-2 fibroblast cell proliferation.

Legend: Figure 21. Proliferation of HFFF-2 fibroblast cells induced by sLDL containing peptide F (0.03) after 5 days incubation in PC-1 serum free media. Level of peptide F present in sLDL 0.03 moles/mole of cholesterol ester. sLDL lipid constituents: cholesterol, cholesterol oleate, triolein and phospholipid.

Figure 22: The effect of the pyrene butyric acid peptide on CHO cell proliferation.

Legend: Figure 22. Proliferation of CHO cells induced by sLDL containing peptide F (0.03) after 5 days incubation in CHO protein free media. Level of peptide F present in sLDL 0.03 moles/mole of cholesterol ester. sLDL lipid constituents: cholesterol, cholesterol oleate, triolein and phospholipid.

The invention will now be illustrated with the following non-limiting examples.

Example 1.

Materials and Methods
Materials.

Egg yolk phosphatidylcholine (PC) was purchased from Lipid Products, Surrey, United Kingdom and used without further purification (>99% pure). Tris(hydroxymethyl)-methylamine (Tris), hydrochloric acid (HCl), dimethylsulfoxide (DMSO) and t-butanol (all Analar grade) were obtained from Merck Ltd, Glasgow, United Kingdom. Triolein (99% pure, TO), cholesteryl oleate (98% pure, CO), phosphate buffered saline (PBS) tablets and the reagents used for cholesterol testing were purchased from the Sigma Chemical Company, Dorset, United Kingdom. The

polycarbonate filters used in the extrusion process were obtained from Costar Corporation, Buckinghamshire, United Kingdom. Sterile, disposable 0.2 µm polysulfone filters used for filter sterilisation were purchased from Whatman Ltd, Maidstone, United Kingdom. Sterile, distilled water complying with the European Pharmacopoeia's water for injection monograph was purchased from Steripak Ltd, Runcorn, United Kingdom. All synthetic polypeptides were synthesised at Thistle Peptide Services (formerly BioMac) at the Department of Biochemistry, University of Glasgow. Peptide structures are listed in Figure 7, material was supplied at greater than 90% purity and was used as received. All tissue culture materials (media, serum, culture flasks, pipettes and multi-well plates) were obtained from Life Technologies Ltd., Paisley, United Kingdom. U937 cells were obtained from the European Collection of Animal Cell Cultures, Salisbury, United Kingdom. DFCS was prepared from FCS by the method of Rothblat et al [6]. Monoclonal anti-LDL receptor antibody (clone C7, code number RPN.537) was purchased from Amersham International and reconstituted with sterile water just prior to use.

METHODS

Preparation of nLDL Particles

Method A

A 3:2:1 molar mixture of Phosphatidylcholine: Triolein: Cholesteryl Oleate (PC:TO:CO) was dissolved in chloroform/methanol 2:1 (V/V) and the solvent removed under a stream of nitrogen. The lipids were then re-dissolved and lyophilised from t-butanol for 24 hours (EF4 Modulyo Freeze Dryer, Edwards High Vacuum, Crawley, United Kingdom), then re-suspended in 0.01M Tris-HCl buffer (pH 8.0) to give a final concentration of 7 to 8 %w/v for extrusion. The lipid

dispersions were sonicated under a stream of N_2 for two hours using a 250W sonicator, centrifuged at 10,000 rpm for 60 minutes (MSE Superspeed 75 Ultracentrifuge, MSE Ltd, London, United Kingdom) and then transferred to the Extruder vessel

5 (Lipex Biomembranes Inc, Vancouver, Canada) which was maintained at 50-55°C throughout. The lipid mixture was successively extruded through polycarbonate filters of pore size 0.1 and 0.05 µm using two stacked filters and at least four extrusions under 60 psig pressure provided by a nitrogen

10 source[8]. Samples of lipid microemulsion were diluted with Tris-HCl buffer to give a cholesterol concentration of approximately 1 mmol/l and heated to 55°C in a stirring water bath. Aliquots of peptide dissolved in DMSO were added under the surface of the stirring microemulsion, control

15 experiments were performed with DMSO alone. The volume of DMSO added in each case was kept below 20µl/ml of microemulsion mixture. The peptide-microemulsion complex was incubated at 55°C for 15 minutes then dialysed overnight against 5 litre PBS. The resulting non-naturally occurring LDL (nLDL) were

20 filter sterilised (0.2µm) and stored at 4°C under N_2 before use.

Analysis for Cholesterol Content of the Method A product.

Microemulsions were assayed for cholesteryl ester content

25 using the method of Allain et al [9]. The Cholesterol Reagent (cholesterol oxidase, cholesterol esterase, horseradish peroxidase, 4-aminoantipyrene, p-hydroxybenzenesulfonate and buffer) was warmed to 30°C in a water bath. To 1.0ml of reagent was added 0.01ml of sample, blank (distilled water) or

30 standard (Cholesterol Calibrator, 200mg/100ml). Reagent-sample mixtures were allowed to incubate at 30°C for 10 minutes. The absorbance of each sample was measured at 500nm on a spectrophotometer (Cecil CE272 Ultraviolet Spectrophotometer).

All readings were complete within 30 minutes after the end of the incubation period. Cholesteryl oleate content was calculated by reference to the cholesterol standard.

5   Method B

A mixture of phosphatidylcholine, cholesterol, cholesteryl ester, triglyceride, and peptide in various molar ratios was dissoved in dichloromethane and mixed prior to addition to an aqueous phase. The aqueous phase consisted of sodium oleate

10   (0.2% w/v)(Sigma-Aldrich, Poole, UK), which was used as an emulsifier. The two phases were mixed in a ratio of 1:9 (organic:aqueous) and sonicated for two minutes. The mixture was then microfluidised at pressures up to 25k psi using an ice-cooled EmusiFlex-C5 (Avestin, Canada) and the organic

15   solvent was removed by evaporation. Different lipid ratios and fatty acid constituents were used to optimise formulations and for all systems final cholesterol and phospholipid content was measured and particle size analysis performed by PCS.

The final system was filtered through a 0.2μm filter and

20   then handled and stored aseptically.

Peptide concentration is expressed with respect to the total cholesterol concentration, for example 0.03 moles of peptide per mole of cholesterol.

Fluorescent systems were prepared as above but with the

25   inclusion of the fluorescent probe 3, 3'-Dioctadecyloxacarbocyanine perchlorate (DiO) at a concentration

of 0.079 mg/ml in lipid dichloromethane solution prior to homogenisation.

Storage

5   The product was stored at 4 °C in the dark in sealed plastic containers. Samples were removed aseptically when required.

Chemical Analysis of Method B product

10   Analysis for Cholesterol Ester Content

      To 1 ml of cholesterol reagent (cholesterol oxidase, cholesterol esterase, horseradish peroxidase, 4-aminoantipyrene, p- hydroxybenzenesulfonate and buffer) was added 0.010 ml of sample, blank (distilled water) or standard
15   (203 mg/ 100 ml). The mixture was incubated at 37 °C for 10 minutes. The absorbance of each sample was measured spectrophotometrically at 500 nm. Cholesterol content was calculated by reference to a cholesterol standard.

20   Analysis for Phospholipid Content

      To 3 ml of phospholipid reagent (phospholipase D, choline oxidase, peroxidase, 4-aminoantipyrine, tris buffer, calcium chloride, phenol) was added 0.020ml of sample or standard (choline chloride and phenol) concentration 300mg/100 ml. The
25   mixture was incubated at 37°C for 10minutes. The absorbance of each sample was measured spectrophotometrically at 505nm. Phospholipid content was calculated by reference to a phospholipid standard.

Analysis for Triglyceride Content

To 1ml of triglyceride reagent (4-aminoantipyrine, 3,5 DHBS, horseradish peroxidase, Microbial GK, microbial GPO, microbial lipoprotein lipase, buffer and sodium azide) was
5 added 0.010 ml of sample, blank (distilled water) or Glycerol standard concentration 250 mg/ 100 ml. The mixture was incubated at 37 °C for 10 minutes. The absorbance of each sample was measured spectrophotometrically at 520 nm. Triglyceride content was calculated by reference to a
10 triglyceride standard.

Analysis for DiO Content

A calibration curve was made with several concentrations of DiO dissolved in methanol. The fluorescence was measure in a
15 fluorescence spectrophotometer (Perkin- elmer 650-40). DiO excitation and emission wavelengths are respectively 484 nm and 507 nm. The sample was diluted in methanol and filtered (0.2 mm). The amount of DiO present in the sample was determined by reference to the calibration curve.
20

Determination of Residual Solvents

Residual solvents were determined by headspace GC analysis using a ThermoFinnegan system. A sample of sLDL was diluted 1:10 with fresh distilled water and 5mL placed in a sample
25 vial. The sample vial was heated to 50 °C for 10 minutes and a 5mL volume of headspace injected onto the column. Suitable standard samples containing known concentrations of $MeCl_2$ were also analysed.

Determination of Osmotic Pressure

Osmotic pressure was determined using an Advanced Instruments Osmometer Model 3D3. The instrument was calibrated using traceable standards before measurement of the
5 sLDL systems.

Determination of Endotoxin Content

Endotoxin content was determined using the gel clot Limulus Amoebocyte Lysate test at a concentration of 0.25EU/mL.
10 Suitable standards and controls were employed during the analysis.

Determination of Viable Microbiological Count

A 1mL sample of sLDL was passed through a 0.45μm membrane
15 filter and the filter washed with sterile Sorenson's buffer. The filter was then aseptically transferred to a tryptone soya agar plate and incubated at 31 °C for 5 days. The numbers of resulting colonies were then noted.

20 <u>Physicochemical Measurements of the Method B product</u>

Size Determination by Photon Correlation Spectroscopy.

Particle size analysis was carried out using photon correlation spectroscopy (Zetasizer 4, Malvern Instruments, Malvern, UK). Before analysis samples were diluted with Tris-
25 HCl buffer (0.01M) and filtered (0.2μm). Sizing measurements were carried out at a fixed angle of 90°. The correlator was operated in parallel mode and the cumulants method of analysis was used to calculate the mean sample size weighted according to the intensity of scattered light (z-average diameter).
30 Since this diameter is weighted strongly in favour of large

particles, Rayleigh theory was used to convert intensity distributions into number distribution.

Zeta Potential Measurement

Samples were diluted 1 in 5 with 0.01M Tris buffer (pH 8.0) and Zeta potential measured at 25 °C using a Zetasizer 4 (Malvern Instruments). The applied voltage was 150V in each case and duty cycling was used to limit the cell current to 20mA.

Transmission Electron Microscopy

Formvar/carbon-coated 200 mesh copper grids were glow discharged and a 10μL droplet of suspension was applied followed by an equal volume of 1% methylamine vanadate (Nanovan) negative stain and the grids immediately dried. Imaging was performed at zero energy loss using a LEO 912 energy filtering electron microscope at 80kV.

Cell Culture.

U937 stock cultures were grown at 37°C in RPMI 1640 media supplemented glutamine (10mM), gentamicin (50μg/ml), fungizone (0.5μg/ml) and 10% (v/v) foetal calf serum (FCS). Cells were maintained in a humidified 5% CO_2 atmosphere between $1-8 \times 10^5$ cells/ml and sub-cultured twice weekly.

NSO (ECACC number 85110503)
NSO stock culture was grown in RPMI 1640 media supplemented with 10% v/v foetal bovine serum, fungizone (50mg/ml) and pen-strep (0.1 mg/ml). Cells were maintained between 3 to 9×10^4 cells/ml, in a humidified 5% CO_2 atmosphere, at 37°C and sub-cultured twice a week.

CHO-K1 (ECACC number 85051005)
CHO stock culture was grown in Ham's F12 media supplemented

with 10% foetal bovine serum, glutamine (2mM), fungizone (50mg/ml) and pen-strep (0.1 mg/ml). Cells were seeded at 1 to 2 x 10^4 cell/cm^2 using 0.25% trypsin-EDTA and maintained in a humidified 5% CO_2 atmosphere, at 37°C and sub-cultured twice

5   a week.

       HFFF2 (ECACC number 86031405)

HFFF2 stock culture was grown in Dulbecco's modified Eagle's media supplemented with 10% v/v foetal bovine

10 serum, glutamine (2mM), fungizone (50mg/ml) and pen-strep (0.1 mg/ml). Cells were seeded at 2 to 3 x 10^4 cell/cm^2 using 0.25% trypsin-EDTA and maintained in a humidified 5% CO_2 atmosphere, at 37°C and sub-cultured twice a week.

15 Growth Assay with peptides A-E

       All growth assays were conducted over 72 hours in 24 well plates unless otherwise indicated. One day before each growth experiment, cells were centrifuged at 1500 rpm for 5 minutes and resuspended in RPMI medium containing 5% (v/v) delipidated

20 foetal calf serum (DFCS) to initiate cholesterol starvation and up regulate LDL receptors. Cells were collected by centrifugation and seeded at 1 x 10^5/well. Appropriate amounts of cholesterol (either LDL, microemulsion or nLDL) were then added after filtration (0.2μm) and the well volume made up to

25 1ml with RPMI containing 5% (v/v) DFCS. After 72 hours an aliquot of each well was diluted with PBS and the number of cells present determined in triplicate by Coulter Counter. Different concentrations were repeated four or eight times depending on the experiment. Three wells per experiment were

30 checked at random before commencement of incubation to ensure a uniform cell density was achieved. This has previously been shown to be a contributing factor in the reproducibility of assay results [7]. In all cases, the mean starting cell densities

of control wells were between 0.95 and 1.05 x 10^5 cells/ml.

Growth Assay with peptide F

Cellular growth assays were conducted in 96 well plates
5   incubated in a hunidified 5% CO_2 atmosphere at 37°C. Media was
prepared containing cells and all the required non-lipid
supplements with the test lipid supplement added to the plate.
A column was set up for each test system and control colums of
serum free media and 10% FCS supplemented media included in
10   every plate.

After the required incubation period MTT solution (5mg/mL in
media) was added in an amount equal to 10% of the media volume.
The plates were then incubated at 37°C in a humidified 5% CO_2
atmosphere for 2 to 4 hours. After the incubation period MTT
15   solubilisation solution (10% TritonX-100 in 0.1NHCl in
isopropanol) was added in an amount equal to the volume of
media in each well. The absorbance of each well was then
measured spectrophotometrically at a 570nm using a Multiskan
Ascent plate reader (Thermo Lab Systems). A background reading
20   at 690nm was also obtained and subtracted from the 570nm
reading. All plates contained a control column of media alone.
Absorbance reading were then compared to a standard curve to
determine cell numbers.

The concentration of sLDL added is expressed as the final
25   total cholesterol (free cholesterol and cholesterol esters)
concentration in the media.   The FCS employed in these
experiment at 10% v/v supplementation provided a cholesterol
concentration in the media of 0.036mg.mL.

Results are expressed as a mean percentage for each column
30   against a 10% foetal calf serum supplemented control system.

Results and Discussion

sLDL particles with peptides A-E

The results presented in Figure 1 demonstrate that the growth of U937 was supported by FCS containing media and to a lesser extent by LDL but not by delipidated FCS or a microemulsion with a similar lipid composition to LDL. This result is consistent with published studies which prove U937 has no ability to synthesise cholesterol and overcomes this by receptor mediated uptake of LDL[3].

To determine whether non-naturally occurring LDL (nLDL) could also support cell growth, U937 cells were incubated with {A}nLDL at a cholesterol concentration equivalent to the optimum LDL concentration which produces maximal cell growth (0.08mmol/1). The results presented in Figure 2 demonstrate that {A}nLDL supported the proliferation of U937 cells *in vitro*. The growth increase was significantly greater (p<0.05) than that produced by microemulsion alone but not statistically different (p>0.05) from that supported by LDL. This strongly suggests nLDL uptake by U937 in a manner similar to LDL and which permits cellular proliferation. This result is due to the presence of the receptor peptide on the particle since control microemulsions do not exhibit this property.

The proliferation of U937 induced by LDL is dose dependent and saturable[3], experiments were therefore conducted to investigate if this was also true for nLDL. In Figure 3 the effects of both increasing nLDL cholesterol concentration and adding DFCS to the media is presented. It can be clearly seen that there is an optimal cholesterol concentration and that DFCS enhanced the proliferation of [Peptide D]nLDL by U937 cells. The maximum DFCS induced increase in cell number was three times that of media containing no serum. The optimum cholesterol concentration of [Peptide D]nLDL in media containing DFCS (0.032mmol/1) is comparable to that of U937 cells grown with LDL 0.026mmol/1) [5]. However, in agreement with

earlier findings using LDL, large concentrations of nLDL produced a reduction in cell number when compared with an untreated control. Optimum nLDL cholesterol concentrations (those which resulted in greatest proliferation) were also

5   increased four fold by DFCS (from 0.008mmol/l to 0.032mmol/l) suggesting that serum promoted more efficient nLDL uptake.

In order to determine the effect of peptide configuration on U937 nLDL uptake, proliferation assays were conducted using five nLDL systems, see Figure 7 for details of each peptide.

10 All the nLDL systems over the range of cholesterol concentrations tested produced a significant proliferation (p<0.05 of U937 cells) (Figure 4) when compared with microemulsion alone. The small proliferation supported by microemulsion without polypeptide at high concentrations may be

15 due to weak non-specific phagocytic activity [10]. However the increases in cell number supported by five different nLDL systems (peptides A-E) strongly suggests that inclusion of the receptor peptide on the particle enhances uptake. Peptide B, C and D systems exhibit the greatest increase in proliferation

20 and these contain either no C-terminal lipid anchor (peptide B and D) or the binding region of Apo B embedded in a 22 amino acid residue chain. The variation between systems indicates that peptide sequence and the presence of a C-terminal lipophilic anchor control the extent of proliferation. The

25 least effective systems, peptides A and E contain either the shortest chain length with both C and N terminal lipid anchors (Peptide A) or a non Apo B binding region sequence of amino acids (Peptide E).

LDL supported similar increases to nLDL preparations

30 containing peptides A, D and E but was three times less than [Peptide B] and [Peptide C]nLDL (Figure 4). This may be due to the viability of commercially obtained LDL. The measured increases were ten times less than those reported by Frostegard

et al [3] at a cholesterol concentration of 0.03mmol/l or by Schewe et al, [4] using 20µg/ml LDL cholesterol and by Sells and Barakat [5] including 10µg/ml in the culture medium. This result illustrates the problem associated with the current

5   requirements to isolate fresh LDL for *in vitro* or *in vivo* experiments.

To further determine that nLDL induced proliferation of U937 cells is due to receptor mediated uptake, cells were incubated with various nLDL preparations (cholesterol concentration

10   0.026mmol/l) and a commercially obtained human anti-LDL receptor antibody. An antibody concentration of 10µg/ml was chosen as this had previously been shown to reduce LDL (0.150mmol/l) supported U937 growth to almost zero [3]. Incubation with the antibody produced a small decrease in the

15   growth of U937 cells grown in media containing each of the nLDL preparations and microemulsion (Figure 5). The decreases for [Peptide A]nLDL [Peptide C]nLDL were statistically significant, however, cell numbers were not reduced to the level of control microemulsion.

20   In order to further investigate the effect of anti-LDL receptor antibody on U937 nLDL uptake, a range of antibody concentrations were tested for 72 hours in media containing [Peptide B]nLDL (0.033mmol/l). [Peptide B]nLDL is the most effective nLDL in promoting U937 growth suggesting a maximal

25   receptor interaction (see Figure 4). Low antibody concentrations (5µg/ml) had no effect on proliferation (Figure 6), however higher concentrations produced a dose dependent, saturable inhibition. Even at antibody saturation (15µg/ml) cell proliferation is significantly more ($p < 0.05$) than control

30   wells without lipid. The large inhibition of cell growth (74%, $p < 0.05$) observed in this study at antibody saturation (15µg/ml) further reinforces the supposition that nLDL induced cellular proliferation is via uptake by the LDL receptor.

The nLDL particles presented in this study exhibit a concentration dependent and saturable ability to induce cellular proliferation of U937 cells in vitro. This effect is stimulated by the presence of DFCS in the media and is
5   inhibited by an anti-LDL receptor antibody. These results illustrate that nLDL mimics the effects of native LDL in the U937 cell proliferation assay and demonstrate that nLDL exhibits receptor competency. A comparison of nLDL systems also demonstrates that control of receptor interaction is
10   possible by alteration of the peptide sequence and by varying the number and location of the lipid anchors.

sLDL particles with peptide F

Three batches of sLDL were prepared by the method described herein. The properties of each batch is described below.

15   In general the batches are similar to sLDL produced using the other peptides A-E. On the whole the sLDL with peptide F has a slightly increased diameter which probably reflects the molecular size of the pyrene butyric acid anchor.

One particular advantage of utilising pyrene butyric acid is
20   that the molecule is fluorescent and all the batches containing peptide F were fluorescent.

Table A lists the physiochemical properties of the sLDL particles which contain peptide F and Table B lists the chemical properties of those particles.

Table A: Pyrene butyric acid containing sLDL physiochemical
            properties.

Batch	Peptide concentration	Zeta potential (mV)	SD	Z average Diam (nm)	SD	Number Diam (nm)	SD
1	0.03	-68	0	260	2.1	80.8	1.82
2	0.015	-70	0.7	245	1.7	140	12
3	0.006	-68	1.0	267	2.0	142	14

Table B: Pyrene butyric acid containing sLDL chemical
            properties.

Batch	Peptide concentration	Cholesterol concentration	Triglyceride concentration (mg/dl)	Phopholipid concentration (mg/dl)
1	0.03	179	643	255
2	0.015	564	484	224
3	0.006	409	300	99

The sLDL containing peptide F is able to stimulate the
proliferation of a variety of cell lines in tissue culture.
The results in Figure 20 illustrate that sLDL formed from the
pyrene containing peptide stimulates the proliferation of NSO
cells up to 500 times greater than FCS and fibroblasts up to
125 times greater (Figure 21). As shown in Figure 22 CHO cells
do not exhibit a similar responsiveness and growth is only
stimulated to about 20 percent of an FCS control. However this
is similar to other sLDL preparations.

As detailed above pyrene butyric acid is fluorescent
and after interaction with sLDL containing a pyrene butyric
acid peptide cells also fluoresce (results not shown)

indicating that the system is assimilated by the cells.

The results demonstrate that the pyrene butyric acid anchor present on the peptide can be any lipophilic group which will pertition into a phospholipid environment. This will produce sLDL particles with the same properties, physiochemical and biological as sLDL produced where the lipid anchor is either retinoic acid, cholesterol or fatty acids.

Example 2.

Materials and Methods

Materials, appropriate preparations of nLDL particles, and analysis of cholesterol content follow the outlines as for Example 1, above.

Photon Correlation Spectroscopy

Particle size analysis was carried out using photon correlation spectroscopy (Zetasizer® Model 4, Malvern Instruments, Malvern, (UK). Before analysis samples were diluted with Tris-HCl buffer (0.01M) and filtered (0.2μm). Sizing measurements were carried out at a fixed angle of 90° to the incident beam. The correlator was operated in parallel mode to allow for more accurate size distribution measurements. The cumulants method of analysis was used to calculate the mean sample size weighted according to the intensity of scattered light (z-average diameter). Since this diameter is weighted strongly on favour of large particles, Rayleigh theory was used to convert intensity distributions into number distributions [12]. The viscosity and refractive index values of pure water were used in the size calculations. All results quoted are the mean and standard deviation of at least 10 measurements.

Zeta Potential Measurement

Samples were diluted 1 in 5 with 0.01M Tris buffer (pH 8.0) and zeta potential measured at 25°C using the Zetasizer® 4 (Malvern Instruments). The applied voltage was 150V in each
5   case and duty cycling was used to limit the cell current to 20mA. All results quoted are the mean and standard deviation of at least 10 measurements.

Results and Discussion

10   The effects of increasing concentrations of the peptides on both diameter and zeta potential are presented in Figure 8. All the peptides act to change measured zeta potential from a negative to a positive value but produce markedly different effects on particle diameter. Below 0.5 moles/mole CO Peptide
15   A has no effect on size but produces an almost linear increase in zeta potential with concentration. Higher concentrations produce size increases but zeta potential plateaus at around +17mV. Peptide B at low concentrations provides a dramatic reversal in zeta potential from around -15mV to +15mV without
20   any change in size. Further concentration increases do not increase zeta potential which remains constant at +15mV but size also remains constant. Peptide C around 0.1 moles/mole CO also produces a reversal of zeta potential without affecting measured diameter, whilst at higher concentrations diameter
25   almost doubles and zeta potential decreases but remains positive.

Zeta potential is a measure of surface charge and the observed results indicate that the peptides are spontaneously locating at the microemulsion droplet surface.

30   At low peptide concentrations (below 0.2 moles/mole CO) where diameter remains unchanged, the zeta potential increases produced by Peptides A and B are roughly equal whilst Peptide C has double the effect.

Peptides containing both anchors (Peptides A and C) produce increases in diameter at low concentrations (0.5 and 0.2 moles /mole CO respectively) whilst Peptide B with only one anchor had no effect at any concentration.

5   Similar effects were noted in native LDL where surfactant induced size increases were related to the molecular size of the lipophilic portion [13]. Absence of the cholesterol anchor does not appear to hinder peptide insertion into the particle since the zeta potentials obtained for Peptides A and B are

10   similar. The increased peptide chain length in Peptide C also appears to have an effect on droplet diameter, which increases at lower concentrations than Peptide A.

The results suggest that the lipid anchors become buried in the microemulsion droplet's outer phospholipid layer whilst the

15   relatively hydrophilic peptide remains on the outer surface.

In Figure 9(a) the effect of pH alterations on the measured zeta potential of various [Peptide A]-nLDL systems is presented.   All exhibit a shift to negative values with increasing pH.

20   [NP]-nLDL (without peptide) also exhibits this trend -10mV over 3 pH units, which is related to changes in the ionic strength of the buffer due to pH adjustment with sodium hydroxide. At 0.532 moles Peptide A/mole CO the zeta potential change is -20mV and at 0.106 moles Peptide A/mole CO -16mV,

25   both greater than the control.

At the extreme alkaline pH tested, all systems, with the exception of the highest Peptide A concentration, have similar zeta potential values. This indicates that ionisation of the peptide has been suppressed and the measured value is that of

30   the microemulsion.

The results indicate that the peptide is exposed on the particle's surface.   Results for three different n-LDL preparations at similar peptide concentrations are presented in

Figure 9(b), all display the trend presented in Figure 9(a).

Figure 9(b) shows the effect of pH on the measured Zeta Potential of various nLDL preparations.

In Figure 10 results on the changes of the measured mean number diameter and zeta potential due to alterations in the microemulsion's surface lipid components are presented. The amphipathic lipid mixtures employed were at proportions relative to those found in native LDL [11].

Both Peptides A and B show a reversal of zeta potential with little change in diameter whilst Peptide C induces large alterations in both parameters. Within different nLDL systems only Peptide A exhibited a significant difference in zeta potential between the lipid formulations even although all have similar values before incorporation.

The results presented illustrate that completely synthetic analogue of LDL containing a receptor binding sequence from Apo-B100 can be prepared. The synthetic peptides employed incorporate into the surface of lipid microemulsions in a concentration dependent manner. The resultant particles have physiochemical properties which are comparable to native LDL, with slightly larger diameter but a controllable zeta potential.

Example 3.

Materials and Methods

B16 cells were obtained from the Dermatology Department of the Western Infirmary, Glasgow, United Kingdom.

Other materials, preparation of nLDL particles and analysis of cholesterol content follow the outlines as for Examples 1 and 2, above.

Incorporation of 14C-Cholesteryl Oleate

The required quantity of 14C-cholesteryl oleate (0.5µCi/mmol CO, supplied dissolved in toluene) was mixed if required with ten times its volume of DMSO prior to addition to stirred nLDL
5   heated at 55°C in a water bath. After incubation for 5 hours the mixture was passed down a Sephadex G-25M gel exclusion chromatography column (bed volume 10ml) eluted with PBS. Fractions (1ml) were collected their absorbance (300nm) and 14C-cholesteryl oleate activity measured. Labelled
10   preparations were filter sterilised (0.2µm) and stored at 4°C under N_2 before use.

Tissue Culture

B16 cells were maintained in RPMI 1640 medium supplemented
15   with 10%v/v FCS incubated at 37°C in a 5% CO_2 in air atmosphere. Confluent cultures were trypsinised, harvested and re-seeded at a dilution of 1 in 20 in fresh medium.

Cell Binding Assays

20   B16 cells were harvested in the log growth phase, washed by centrifugation and re-seeded into 24 well plates 1×10^5 cells/ml in media containing 10v/v DFCS. After overnight incubation the cells were cooled to 4°C for 1 hour, the media aspirated and replaced with ice cold media containing 10%v/v
25   DFCS, supplemented with 14C-nLDL at a cholesterol concentration of 0.08mmol/l. Four replicate wells were used for each concentration. Cells were then incubated at 4°C for 4 hours with occasional rocking, the media aspirated and cells washed three times with ice cold PBS/BSA buffer (2 mg/ml BSA in PBS)
30   followed by two ten minute incubations with ice cold PBS/BSA. Three final washes with ice cold PBS were then conducted. The 14C-cholesteryl oleate was extracted with hexane/isopropanol for 30 minutes at room temperature, aspirated and then mixed

with 3 ml of scintillation fluid prior to counting. Cellular protein left in the well after extraction was assayed using Peterson's modification of the Lowry method[14]. Sodium deoxycholate (1.5mg/ml) was added to solubilise the protein,

5   which was then precipitated by the addition of trichloroacetic acid (72%w/v), pelleted by centrifugation (5,000rpm 10 minutes), the supernatant discarded and the pellet solubilised in Lowry reagent at room temperature for 20 minutes. Folin Ciocalteu's reagent was added, the solutions mixed and the

10   colour allowed to develop for 30 minutes prior to absorbance measurements at 690nm using a plate reader. A calibration curve was plotted from BSA standards ranging from 20 to 200μg/ml.

15   Results and Discussion

To determine if nLDL would bind in a receptor dependent fashion, increasing amounts of radio-labelled [Peptide X]nLDL was added to B16 *in vitro* and the results are presented in Figure 11. For four of the [Peptide X]nLDL particles (Peptide

20   B, C, D and E) a saturable increase in total binding was obtained which is typical of receptor ligand interactions. [NP]nLDL (nLDL containing no peptide (NP)) also exhibited saturation binding whilst [Peptide A]nLDL provided a linear response at the concentrations tested. Clearly the choice of

25   peptide incorporated in nLDL has a large effect on the interaction with B16 cells, since [Peptide D]nLDL had almost twice the binding of [Peptide C]nLDL at receptor saturation. Clearly the results indicate that interaction between [Peptide X]nLDL and B16 cells is occurring and that this is moderated by

30   the incorporated peptide.

LDL receptor binding is comprised of two components, high affinity specific binding and non-specific binding (defined as ligand bound in the presence of an excess of unlabelled

ligand)[16]. The former is routinely used for calculation of affinity constants and is determined by subtracting non-specific from total binding.

The non-specific and specific binding determined for various
5   [Peptide X]nLDL preparations is presented in Figures 12(a) and 12(b). These results illustrate that for all the concentrations tested the specific binding of [Peptide B], [Peptide D] and [Peptide E]nLDL was higher than that obtained for either [NP], [Peptide C]nLDL. In addition [Peptide B],
10   [Peptide D] and [Peptide E]nLDL exhibit higher specific than non-specific binding.

Double reciprocal plots are presented in Figure 13 and the calculated constants in Table 1. For all preparations linear regression analysis provided correlation coefficients which
15   were statistically significant (See Table 1). The remaining [Peptide X]nLDL preparations have a gradation of binding constants [Peptide E] > [Peptide B] > [Peptide D]. Since [NP]nLDL provided a saturation binding curve it also provided a binding constant with a value very similar to [Peptide
20   D]nLDL.

These results demonstrate that nLDL exhibits saturable binding kinetics with B16 cells *in vitro* at 4°C, with the extent of binding determined by the incorporated peptide's structure. An eleven amino acids peptide with two lipid
25   anchors produces binding [Peptide A], however removal of the C-terminal cholesterol significantly improves performance [Peptide B]. A twenty two amino acid long peptide further enhances binding [Peptide C] and similarly removal of the cholesterol anchor results in further increases in binding
30   competence [Peptide D]. This improvement can be modulated by using a random sequence [Peptide E] based on the amino acids present in the previous peptides. This strongly indicates that the measured binding is related to the peptide and its

structure (length, lipid anchor and sequence) rather than some simple physical effect.

Table 1: Binding constants of [Peptide X]nLDL determined
from double reciprocal plots of total binding

Preparation	Kd (x10^{-4}M)	Δmax (x10^{-5}M)
[NP]nLDL	3.2	7.86
[Peptide A]nLDL	*	*
[Peptide B]nLDL	9.3	30.6
[Peptide C]nLDL	7.7	17.2
[Peptide D]nLDL	3.0	18.5
[Peptide E]nLDL	15.2	32.4

Values calculated using linear regression, all lines statistically significant $p < 0.05$.

* Not calculated since intercept positive.

Example 4.

Materials and Methods

Materials, preparation of microemulsion and of nLDL particles, and analysis of cholesterol content are as for Example 1, above with the exception that B16 cells are used in place of U937 cells.

Fluorescent Labelling of Microemulsion, nLDL.

LDL, nLDL and microemulsion were labelled with DiO by a method modified from Stephan and Yuracheck [18] (as described

hereinbelow). A fluorescent probe stock solution (3 mg/5ml in DMSO) was added to LDL solution at a concentration of 300 µg/mg LDL protein or to microemulsion or nLDL suspension containing an equivalent concentration of cholesterol (1mmol/l) in an identical volume. The lipid DiO mixture was incubated in the dark at 37°C for five hours then passed down a Sephadex G-25M column (bed volume 9.1ml) eluted with PBS. The resulting labelled nLDL were filter sterilised (0.2µm) and stored at 4°C under N_2 before use. The DiO concentration of the microemulsions was determined using a fluorescence spectrophotometer (Perkin Elmer 203, Xenon Model 150 lamp) and excitation and emission wavelengths of 484 and 587nm respectively. Samples of DiO labelled material (200µl) were added to methanol (3ml), fluorescence determined and concentration calculated with reference to a standard curve.

Cell Culture.

B16 cells in log phase were trypsinised and inoculated into 24 well plates at a concentration of 5×10^4 cells per well containing RPMI 1640 (1ml) supplemented with 10% v/v FCS. Cells were left to grow 24 hours then serum starved in RPMI 1640 supplemented with 5% v/v DFCS for a further 24 hours. DiO labelled microemulsion and nLDL were filtered (0.2µm) and added to wells at known concentrations. Plates were then incubated for three hours at either 4°C to determine bound or 37°C to determine cell associated DiO concentrations. Plates incubated at 4°C were chilled for 30 minutes prior to lipid addition. After incubation remaining media was aspirated, the cells carefully washed to prevent detachment then methanol (1ml) added to each well and incubated at room temperature with gentle shaking for 30 minutes. The methanol extract was centrifuged at 5,000rpm for 5 minutes, aspirated and added to methanol (2ml) and the DiO concentration determined by

fluorimetery as previously described. Cells incubated without DiO were also methanol extracted to account for auto-fluorescence. Three wells per plate were routinely assayed for protein concentration by adding 2M NaOH and using Peterson's [14]

5   modification of the Lowry method [15]. B16 cells are contact inhibited and were near confluency during the experimental procedure, the mean value from this determination was utilised. Specifically bound and associated DiO was determined by including a hundred fold excess concentration of non-labelled

10   ligand in two wells for each concentration measured. Specific binding was calculated as total binding (without excess) less non-specific binding (with excess). Data was treated using a Scatchard and Double Reciprocal Analysis to calculate binding constants.

15

Results

To investigate if nLDL could be labelled for use in receptor binding studies, samples were chromatographed using a Sephadex G-25M column (PD-10). The fractions were monitored for DiO and

20   cholesterol content and results are presented in Table 2.

To determine if the DiO was firmly bound to nLDL, samples were incubated with identical volumes of PBS containing Bovine Serum Albumin (BSA, 2mg/ml) for 30 minutes at 37°C and then chromatographed using a Sephacryl S-300 column. Over eighty

25   percent of the fluorescence was associated with the particle (Figure 14) and only a small amount co-eluted with the protein.

Carbocyanine dyes such as DiO are known to show little movement after insertion into a phospholipid layer especially if cholesterol is also present.

30   The data demonstrates that nLDL suspensions can be labelled with DiO using a modification of the method previously devised for LDL [17], [18].

Initial studies on the binding of DiO labelled nLDL to B16 cells were performed without an excess of unlabelled ligand. The original nLDL prepared was not concentrated enough to enable the sufficient excess (100 times labelled) to be added

5   to small volume culture wells. [Peptide A]nLDL and microemulsion displayed similar binding profiles (Figure 15), however [Peptide C]nLDL saturated at higher levels of added ligand. Total binding curves of DiO labelled nLDL to B16 cells at 4°C appear to be dose dependent and saturable suggesting a

10   receptor mediated interaction.

Figure 16 shows the effect of increasing cholesterol concentration on the binding (specific, non-specific and total) of labelled [Peptide A]nLDL and microemulsion to B16 cells at 4°C. Both had similar total binding curves but microemulsion

15   exhibited an increased non-specific component when compared with [Peptide A]nLDL. [Peptide A]nLDL non-specific binding although linear and therefore proportional to the amount added was greater than calculated specific binding. [Peptide A]nLDL specific binding appears to be concentration dependent but at

20   the concentrations examined was not saturable.

The amount of nLDL internalised in three hours at 37°C can be calculated by subtracting total bound from total cell associated (Figure 17). [Peptide A]nLDL produces a linear dose response for all three parameters over a cholesterol range of

25   0 to 25µmol/l, suggesting that even the highest concentration of [Peptide A]nLDL was not saturating the receptor dependent uptake.

These results show that [Peptide A]nLDL can readily bind to LDL receptors.

30   Receptor binding constants were calculated from Scatchard and Double reciprocal plots (Figure 18(a) and 18(b) and are shown in Table 3. Both types of analysis resulted in much higher association constants for [Peptide A]nLDL and [Peptide C]nLDL

when compared with microemulsion.

The results highlight the differences between particle with and without peptide and between the peptides. Specific binding appears to be conferred by the presence of the peptide and the
5 degree of binding is controlled by peptide structure.

The results indicate that the inclusion of an amphipathic peptide containing the ApoB receptor sequence onto nLDL particles confers high affinity binding to a cell surface receptor and that binding affinity is influenced by peptide
10 length. Doubling peptide lengths from 11 to 22 amino acids almost doubles the measured affinity.

This result coupled with the data presented in Example 1 indicates that [Peptide X] nLDL particles interact with the Low Density Lipoprotein receptor on B16 cells. This interaction is
15 controlled by the amphipathic peptide's overall length and the number of lipophilic anchors; two anchors produce a lower binding than a single one.

Table 2: Recovery of cholesterol and DiO from PD-10
20   elutions of LDL and nLDL.

	Starting Concentration nLDL	Total Recovered	Percentage Recovered nLDL
Cholesterol	2.8 µmol	2.4 µmol	96%
DiO	600 µg	124.6 µg	21%

25

Table 3: Receptor Binding Constants determined from Scatchard and Double Reciprocal Analysis.

	Scatchard			Double Reciprocal		
Sample	Kd	k	Bmax	Kd	k	Bmax
Microemulsion	1.28	0.78	0.028	6.92	0.14	0.092
[PeptideA]nLDL	0.53	1.9	0.026	1.17	0.85	0.037
[PeptideB]nLDL	0.37	2.7	0.081	0.70	1.43	0.093

Example 5.

Materials, and methods of preparation of nLDL particles, analysis of cholesterol content, cell culture and growth or proliferation assay are as described for Example 1. Photon correlation spectroscopy and zeta potential measurement are as described in Example 2.

Results

Several studies have suggested that estrogens exert a hypolipidaemic effect via upregulation of LDL receptor numbers and subsequent enhanced plasma LDL clearance [19]. If nLDL induced proliferation of U937 occurs via the LDL receptor the addition of estrogen to the media should enhance this effect. The results of estrogen treatment on induced proliferation is presented in Figure 19. Estrogen has no effect on control systems without peptide but produces a dramatic increase in proliferation for [Peptide A]nLDL and [Peptide B]nLDL at similar peptide concentrations. The estrogen stimulated [Peptide B]nLDL proliferation at higher peptide concentrations is less and is completely lost at the highest value tested. The significantly increased U937 proliferation achieved

suggests that these systems do utilise the LDL receptor pathway.   The lack of estrogen stimulation of [NP]nLDL indicates that the effect cannot be ascribed to increased phagocytosis or some other similar non-specific mechanism.

5   Estrogenic stimulation of proliferation is further indication that nLDL uptake is via the LDL receptor pathway. nLDL systems are indicated as being useful as controllable LDL substitutes for application to drug targeting tasks.

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